Common Grounds

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Summary

This thesis is about the concept that chemical contaminations present in soils can cause stress in soil-dwelling organisms, which becomes evident in specific gene transcripts in the cells of these organisms. The research presented here focused on the development of a method that would apply gene expression quantification using reverse transcription quantitative PCR (RT-qPCR) to ecotoxicological assessment of soil quality. A standard ecotoxicological model species was used, namely the springtail *Folsomia candida*.
Standardized bioassays using terrestrial invertebrates are a key component of risk assessment of soil. These assays take bio-availability of soil-based chemicals into account and thus quantify the actual risks associated with the tested soil rather than a predicted estimate based on chemical analyses. Molecular biomarkers can provide added value to current risk assessment. This is particularly the case for light contaminations, as they are ultra-sensitive. Also they can give insight in the nature of the encountered stress, since molecular responses to different classes of chemicals will differ. The main aim of this thesis was to assemble a set of markers that could indicate and predict different types and levels of chemical stress, on the basis of differences in gene expression profiles of exposed animals.

The second chapter describes the first steps of developing the molecular indication method. These steps were to acquire and validate differentially expressed gene transcripts of interest. Two model compounds from different chemical classes, the metal cadmium (Cd) and the polycyclic aromatic hydrocarbon (PAH) phenanthrene, were selected for initial identification of stress-related transcripts. cDNA stress-libraries were constructed using suppressive subtractive hybridization (SSH). A test set of 44 bio-indication gene assays were selected from the SSH libraries. A set of independent samples of two different exposure concentrations of the same chemicals, cadmium and phenanthrene, was used to validate the test set of assays. Analysis of the experiment was carried out on a novel high-throughput qPCR platform, which makes use of gene expression chips that are able to quantify 48 × 48 = 2304 reactions per run. The analytic power of measuring 48 assays in 48 samples in about three hours time is well-suited for this type of ecotoxicological experiments where testing of large numbers of samples is to be preferred.
The SSH library for cadmium contained transcripts related to mitochondrial dysfunction and protein degradation, while for phenanthrene the majority of transcripts present in the library were related to biotransformation. Using multivariate partial least squares discriminant analysis (PLS-DA) it was possible to classify the samples according to exposure type by ranking the classification scores.

The third chapter describes the validation of reference genes for the purpose of RT-qPCR normalization. Transcription levels in gene expression studies are commonly expressed 'relatively'; in most cases relative to the control samples. To aim for a normal distribution of the data the expression levels are usually also depicted in a $\log_2$ scale or as 'fold change'. A gene could for example be "2 fold upregulated in exposed samples compared to control". This means that the amount of this particular mRNA is twice as much in the exposed samples than in the controls. The first step to calculate transcription levels though, is a normalization with an internal reference. Often, the transcription levels of one or more stable 'reference' genes are used for this purpose. Normalization is needed to correct for factors such as RNA input differences and reverse transcriptase efficiency variation. An essential requirement for reference genes is the stability of their transcriptional level across the various conditions to which an organism is exposed during an experiment. Therefore, the use of multiple reference genes rather than one, and beforehand validation of the selected gene set is advisable.
Assays for candidate reference genes were developed for two Collembola species, *Folsomia candida* and *Orchesella cincta*, and were tested for six (xenobiotic and environmental) treatments. Both springtail species are used regularly in RT-qPCR experiments, and therefore the broader purpose of this experiment was to create a foundation for future environmental genomic studies. The stability of each reference gene over the array of differently treated and untreated samples was evaluated using two common methods for reference gene validation; geNorm and Normfinder. The results demonstrated a difference in stability of genes between treatments, indicating that there are no generic reference genes and specific reference genes should be chosen when studying different stresses. Reference gene suitability also differed between species, even when similar stresses were applied. For the quantification of responses to chemical stressors in *F. candida*, particularly to the compounds cadmium and phenanthrene, the use of two reference genes, *YWHAZ* and *SDHA*, was found to be the optimal set.

The fourth chapter describes an experiment where binary mixtures of cadmium and phenanthrene are first assessed by a standard ecotoxicological bioassay, reproduction of *Folsomia candida*, and then evaluated by transcriptional profiling using a set of 86 target genes.
In recent years, an important shift in perspective has taken place in ecotoxicology: from the initial focus on the stressors, e.g. the chemical compounds present in the soil, towards a focus on the responses invoked inside the organism as a consequence of exposure to stressors. The study of mixture toxicity has benefited greatly from this paradigm shift: A mechanistic understanding on how chemicals interact in regard to occurring responses in the organism, enhances interpretation of the ecotoxicological effects. The knowledge on dose-response relationships of individual chemicals to

endpoints such as growth, survival or reproduction, can form the basis to interpret the combined effects. Therefore, the linking of gene expression profiles to established ecotoxicological relevant endpoints is essential. Making this linkage is still one of the challenges today.
The aim of the mixture experiment was to gain understanding of the molecular mechanisms underlying the observed responses on reproduction. These responses showed an antagonistic interaction between cadmium and phenanthrene: The mixture exposures impacted reproduction to a lesser extent than could be expected on the basis of the effects the single chemicals exposures. Transcriptional response profiling using PLS regression reflected the global responses to both the individual chemicals, but also distinct mixture responses were inferred. It appeared that oxidative stress levels encountered during the early exposure period remained lower in the mixture treated samples compared to those of the single compounds. This may to a degree have accounted for the antagonistic mixture effects found on reproduction.

In the fifth chapter, this mixture mechanism is investigated further. The temporal patterns of the transcriptional changes were studied by assessing a time series over the first two weeks of exposure. Exposure to cadmium only (EC_{50}; reproduction) was compared to a mixture of cadmium with an added low concentration of phenanthrene.
Transcription profiles of the cadmium and the mixture exposures were more alike after 7 days than after shorter exposure times. Early response patterns differed between the treatments mainly because of a prominent induction of biotransformation gene assays in the mixture treatment. Over time, the single cadmium exposure was distinguished from the mixture treatment by differences in patterns of assays that relate to the vesicular deposition of cadmium.

The sixth chapter describes a case study of a former landfill area in the Netherlands where a bioremediation project is running, in order to redevelop the area for recreational purposes. A complex mixture of chemicals was present in the soil and standard toxicity tests revealed that a dilution of 16% sample soil with 84% clean soil caused 50% inhibition of reproduction upon exposure. Toxicity tests were also used to assess a HPLC extract of the test soil, holding the organic compounds that were present in the soil but not, for instance, the water soluble metals. The impact of the extract to reproduction of *F. candida* was much lower than that of the soil.
A subsequent microarray analysis of two concentrations from each sample type was done; the ‘no effect’ and the 50% effect dilutions. A principal component analysis was able to distinguish soil samples from extract samples, and could classify the two concentrations of the extract. A differentiation between the measured concentrations of soil samples however, could not be defined. The dilution series of the extract and soil were further used to test for dose dependency of the responses using a subset of

the assays from the RT-qPCR method. Dose-dependent responses were found for cytochrome P450 assays that are involved in phase I of the biotransformation of xenobiotics. This opens the possibility to link gene expression levels to adverse effects at the organismal level.

The final chapter contains a general discussion of the findings in the context of the current knowledge of stress response pathways, particularly the oxidative stress response and the unfolded protein response (UPR) of the endoplasmic reticulum (ER), the interconnected membrane network within cells. These two systems get activated by different triggers, but are also closely linked. Crosslinks between the systems are common regulators such as Nrf2 and NFκB. It makes sense that such connections exist because cellular effects of xenobiotic compounds permeating cells are often multi-faceted. Also, the processes that get activated to deal with the potentially harmful conditions can cause stress by themselves.
The two chemicals that were studied in detail, cadmium and phenanthrene, most likely trigger each of these stress response systems in a different way. Cadmium causes oxidative stress. In combination with phenanthrene however, the oxidative stress levels probably remain lower during the early phase of exposure to cadmium. This may be related to a feedforward trigger via the onset of biotransformation. This could enhance the cellular glutathione status, which can increase the protection from Cd^{2+} initiated oxidant injury.

The application of these findings in ecotoxicological assessments of soils is manifold. Risk assessment could already benefit from the developed molecular biomarkers, with respect to gaining insight in the nature of exposure. The *MT2* as well as some of the cytochrome P450 assays were class specific. The common classes of metals and PAHs could thus be identified by using such markers. These assays are also suitable for use in the low concentration ranges that occur in the environment.
One of the key findings was that the expression profiles were more apt to discriminate and predict the nature of a chemical exposure than a stress level. Some assays showed a concentration-related response, but the majority of transcriptional responses were not simple dose-dependent relationships. The severity of the encountered stress levels however, can often be distinguished by the nature of the induced transcripts. At light and intermediate levels of oxidative stress, the pathway dynamics promote restoration of the cells, while for intermediate to severe stress levels, the pathways tend to be associated with apoptosis and cell death. The concept of using such fluctuations in the nature of the induced transcripts opens up possibilities for multi-assay assessment of stress levels.

The work described in this thesis forms an early stage in the development of an integrated ecotoxicological systems biology approach for soil quality assessment. Biotechnological developments of RNA sequencing already made the discovery of relevant transcripts much easier. Obtaining the key nodes within the pathways of interest will therefore also become easier, which could benefit the efficacy of the type of method as it is presented here. Future work on stress response profiling will progress from the current stage of discovery of new phenomena, towards hypothesis-driven research. For this purpose, high throughput RT-qPCR is perfectly well-suited.